Manual

MutaPLEX® Borrelia real time PCR TM kit

Test for the qualitative in-vitro detection of DNA of Borrelia burgdorferi sensu lato in clinical specimens and ticks

Valid from 2018-11-19

REF **KG1917032** Σ 32

REF **KG1917096** Σ 96

-20 °C

IVD

CE

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1 INTENDED USE

The MutaPLEX® Borrelia real time PCR TM kit is an assay for the detection of DNA of *Borrelia burgdorferi sensu lato* in clinical specimens and ticks using real time PCR microplate systems.

2 PATHOGEN INFORMATION

Borrelia are gram-negative bacteria of the spirochaete family. Members of the genus *Borrelia* are the causative agents of two important tick-borne diseases: relapsing fever and Lyme disease.

In Europe, Lyme borreliosis is the most common vector-borne disease. The highest incidence is reported from Austria, Switzerland, the Czech Republic, Germany, Slowenia, as well as from the northern countries bordering the Baltic Sea.

Lyme borreliosis is a multi-system disorder, which can lead to severe complications of the neurological system, the heart and the joints. At an early stage of its manifestation, borreliosis is treatable with antibiotics, however, clinical diagnosis is complicated. Antibodies are not detectable in the blood until weeks after infection and symptoms are highly variable.

Analysis of ticks offers the possibility to identify the risk of infection very quickly, and therefore minimising the delay of an antibiotics treatment.

3 PRINCIPLE OF THE TEST

The MutaPLEX® Borrelia real time PCR TM Kit contains specific primers and dual-labeled probes for the amplification and detection of *Borrelia burgdorferi sensu lato* DNA in clinical specimens and ticks.

The presence of nucleic acid is detected by an increase in fluorescence due to hydrolysis of the probes during amplification.

The fluorescence of the pathogen-specific probes is measured in the FAM channel.

Furthermore, MutaPLEX® Borrelia real time PCR TM Kit contains a control DNA, which is added during DNA extraction and detected in the same reaction by a differently labeled probe.

The control DNA allows the detection of PCR inhibition and acts as control, that the nucleic acid was isolated from the clinical specimen.

The fluorescence of the control DNA is measured in the VIC®/HEX/JOE/TET channel.

4 PACKAGE CONTENTS

The reagents supplied are sufficient for 32 (KG1917032) or 96 (KG1917096) reactions, respectively.

Table 1: Components of the MutaPLEX® Borrelia real time PCR TM kit .

Label	Lid Colour	Content	
		32	96
Reaction Mix	yellow	1 x 512 µl	2 x 768 µl
Positive control	red	1 x 50 µl	1 x 100 µl
Negative control	green	1 x 50 µl	1 x 100 µl
Control DNA	colourless	1 x 160 µl	2 x 240 µl

5 EQUIPMENT AND REAGENTS TO BE SUPPLIED BY USER

- DNA isolation kit (e. g. MutaCLEAN® Universal RNA/DNA, KG1038, or the magnet particle based system MutaCLEAN® Mag RNA/DNA, KG1023/KG1024)
- PCR grade water
- Sterile microtubes
- Pipets (adjustable volume)
- Sterile pipet tips with filter
- Table centrifuge
- Vortex mixer
- Real time PCR instrument
- Optical PCR reaction tubes with lid
- Optional: Liquid handling system for automation

6 TRANSPORT, STORAGE AND STABILITY

The MutaPLEX® Borrelia real time PCR TM kit is shipped on dry ice. All components must be stored at maximum -20 °C in the dark immediately after receipt. Do not use reagents after the date of expiry printed on the package.

Up to 20 freeze and thaw cycles are possible.

For convenience, opened reagents can be stored at 2–8 °C for up to 6 months.

Protect kit components from direct sunlight during the complete test run.

7 IMPORTANT NOTES

- The MutaPLEX® Borrelia real time PCR must be performed by qualified personnel only.
- Good Laboratory Practice (GLP) has to be applied.
- Clinical samples must always be regarded as potentially infectious material and all equipment used has to be treated as potentially contaminated.

8 GENERAL PRECAUTIONS

- Stick to the protocol described in the instructions for use.
- Set up different laboratory areas for the preparation of samples and for the set up of the PCR in order to avoid contaminations.
- Pipettes, tubes and other materials must not circulate between those different laboratory areas.
- Always use filter tips.
- Regulary decontaminate equipment and benches with ethanol-free decontaminant.
- Do not combine MutaPLEX® Borrelia real time PCR TM kit components of different lot numbers.

9 SAMPLE MATERIAL

Starting material for the MutaPLEX® Borrelia real time PCR is DNA isolated or released from clinical specimens (e. g. EDTA blood, plasma, serum, cerebrospinal fluid and tissue samples) or from ticks.

10 SAMPLE PREPARATION

The MutaPLEX® Borrelia real time PCR is suitable for the detection of *Borrelia burgdorferi sensu lato* DNA isolated from clinical specimens or ticks with appropriate isolation methods.

Commercial kits for DNA isolation such as MutaCLEAN® Universal RNA/DNA (KG1038) or the magnet particle based system MutaCLEAN® Mag RNA/DNA (KG1023/KG1024) are recommended.

It is recommended to use mechanical disruption of ticks before DNA extraction. Please follow the instructions for use of the respective extraction kit.

Important: In addition to the samples, always run a water control in your extraction. Treat this water control analogous to a sample.

Comparing the amplification of the control DNA in the samples to the amplification of the internal control in the water control will give insights on possible inhibitions of the real time PCR. Furthermore, possible contaminations during DNA extraction will be detectable.

Please note chapter 11 "Control DNA".

If the real time PCR is not performed immediately, store extracted DNA according to the instructions given by the DNA extraction kit's manufacturer.

11 CONTROL DNA

A control DNA is supplied and can be used as extraction control or only as inhibition control. This allows the user to control the DNA isolation procedure and to check for possible real time PCR inhibition.

DNA isolation from EDTA blood, plasma, serum, cerebrospinal fluid, tissue samples and ticks

a) Control DNA used as extraction control

MutaPLEX® Borrelia control DNA is added to the DNA extraction.

Add 5 µl control DNA per extraction (5 µl x (N+1)). Mix well. Perform the DNA isolation according to the manufacturer's instructions. Please follow protocol A.

The control DNA must be added to the lysis buffer of the extraction kit.

b) Control DNA used as internal control of the real time PCR

If only inhibition will be checked, please follow protocol B.

12 REAL TIME PCR

12.1 Important points before starting

- Please pay attention to chapter 7 "Important Notes".
- Before setting up the real time PCR, familiarise yourself with the real time PCR instrument and read the user manual supplied with the instrument.
- The programming of the thermal profile should take place before the PCR set up.

- In every PCR run, one positive control and one negative control should be included.
- Before each use, all reagents except the enzyme should be thawed completely at room temperature, thouroughly mixed, and centrifuged very briefly.
- We recommend to keep reagents and samples at 2–8 °C (e. g. on ice or a cooling block) at all times.

12.2 Procedure

If the control DNA is used to control both, the real time PCR and the DNA isolation procedure, please follow protocol A. If the control DNA is solely used to detect possible inhibition of the real time PCR, please follow protocol B.

Protocol A

The control DNA was added during DNA extraction (see chapter 11 "Control DNA"). In this case, prepare the master mix according to table 2.

The master mix contains all of the components needed for PCR except the sample. Prepare a volume of master mix for at least one sample more than required, in order to compensate for pipetting inaccuracy.

Table 2: Preparation of the master mix (control DNA was added during DNA extraction)

Volume per reaction	Volume master mix
16 µl Reaction mix	16 µl x (N+1)

Protocol B

The control DNA is used for the control of the real time PCR only (see chapter 11 "Control DNA"). In this case, prepare the master mix according to table 3.

The master mix contains all of the components needed for real PCR except the sample. Prepare a volume of master mix for at least one sample more than required, in order to compensate for pipetting inaccuracy.

Table 3: Preparation of the master mix (control DNA is added directly to the master mix)

Volume per reaction	Volume master mix
16 µl Reaction mix	16 µl x (N+1)
0.5 µl Control DNA*	0.5 µl x (N+1)*

* The increase in volume caused by adding the control DNA is not taken into account when preparing the PCR assay.

Protocol A and B: real time PCR set up

- Place the number of optical PCR reaction tubes needed into the respective tray of the real time PCR instrument.
- Pipet 16 µl of each master mix into each optical PCR reaction tube.
- Add 4 µl of the eluates from the DNA isolation (including the eluate of the water control), the respective positive control and the respective negative control to the corresponding optical PCR reaction tubes (table 4).
- Close the optical PCR reaction tubes immediately after filling in order to reduce the risk of contamination.

Table 4: Preparation of the real time PCR

Component	Volume
Master mix	16.0 µl
Sample	4.0 µl
Total volume	20.0 µl

12.3 Instrument settings

For the real time PCR, use the thermal profile shown in table 5.

Table 5: real time PCR thermal profile

Description	Time	Temperature	No of cycles
Initial denaturation	10 min	95 °C	1
Amplification of DNA			45
Denaturation	10 s	95 °C	
Annealing	20 s	60 °C	
	Aquisition at the end of this step		
Extension	10 s	72 °C	

If in the same run samples should be tested for pathogens with RNA genome, e.g. with the MutaPLEX® FSME (TBE) real time RT-PCR kit, use the thermal profile shown in table 6.

Table 6: real time RT-PCR thermal profile

Description	Time	Temperature	No of cycles
Reverse transcription	20 min	45 °C	1
Initial denaturation	5 min	95 °C	1
Amplification of DNA			45
Denaturation	10 s	95 °C	
Annealing	20 s	60 °C	
	Aquisition at the end of this step		
Extension	10 s	72 °C	

Dependent on the real time instrument used, further instrument settings have to be adjusted according to table 7.

Table 7: Overview of the instrument settings required for the MutaPLEX® Borrelia real time PCR.

Real time PCR Instrument	Parameter	Detection Channel	Notes	
LightCycler 480I	Borrelia Control DNA	483–533 523–568	pre-installed universal Color Compenation FAM (510) – VIC (580)	
LightCycler 480II	Borrelia Control DNA	FAM (465–510) HEX (533–580)		
Stratagene Mx3000P/ Mx3005P	Borrelia Control DNA	FAM HEX	Gain 8 Gain 1	Reference dye: none
ABI 7500	Borrelia Control DNA	FAM JOE	Option reference dye ROX: NO	
Rotor-Gene Q/3000/ 6000	Borrelia Control DNA	Green Yellow	Gain 5 Gain 5	
Mic qPCR Cycler	Borrelia Control DNA	Green Yellow	Gain 8 Gain 10	

13 DATA ANALYSIS

The *Borrelia*-specific amplification is measured in the FAM channel. The amplification of the control DNA is measured in the VIC®/HEX/JOE/TET channel.

The following results can occur:

A signal in the FAM channel is detected:

The result is positive, the sample contains Borrelia DNA.

In this case, detection of a signal of the control DNA in the VIC®/HEX/JOE/TET channel is inessential, as high concentrations of bacterial DNA may reduce or completely inhibit amplification of the control DNA.

No signal in the FAM channel, but a signal in the VIC®/HEX/JOE/TET channel is detected:

The result is negative, the sample does not contain Borrelia DNA.

The signal of the control DNA excludes the possibilities of DNA isolation failure (in case the control DNA is being used as an extraction control) and/or real time PCR inhibition. If the CT value of a sample differs significantly from the CT value of the water control, a partial inhibition occured, which can lead to negative results in weak positive samples (see chapter "Troubleshooting").

Neither in the FAM channel nor in the VIC®/HEX/JOE/TET channel a signal is detected:

A diagnostic statement cannot be made.

The DNA isolation was not successful or an inhibition of the PCR has occurred. In case the control DNA was added during DNA isolation and not directly to the PCR master mix, the negative control is negative in both channels.

Figure 1 and figure 2 show examples for positive and negative real time PCR results.

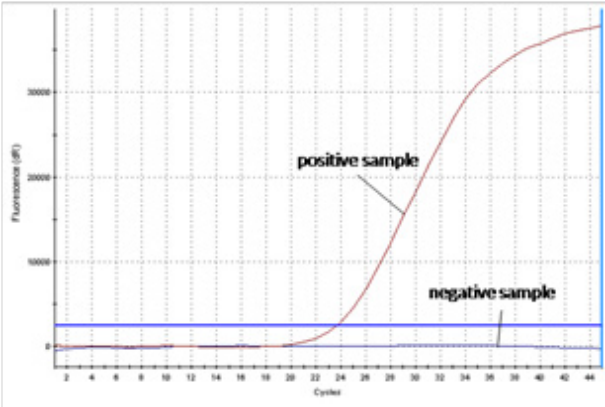

Figure 1: The positive sample shows bacteria-specific amplification, whereas no fluorescence signal is detected in the negative sample.

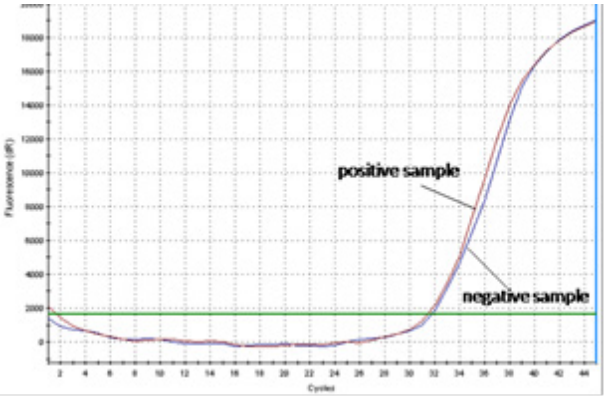

Figure 2: The positive sample as well as the negative sample show a signal in the control DNA-specific VIC®/HEX/JOE/TET channel. The amplification signal of the control DNA in the negative sample shows, that the missing signal in the bacteria-specific channel is not due to RT-PCR inhibition or failure of DNA isolation, but that the sample is a true negative.

14 ASSAY VALIDATION

Set a threshold as follows:

Negative controls

All negative controls should be below the threshold. If there is a potential contamination (appearance of a curve in the negative control or a cluster of curves in speci-

mens at high C_T – for example above 36), results obtained are not interpretable and the whole run (including extraction) has to be repeated.

Positive controls

All the positive controls must show a positive (i.e. exponential) amplification curve. The positive controls must fall below a C_T of 30.

Internal controls

All internal controls must show a positive (i.e. exponential) amplification curve. The internal control must fall below a C_T of 33. If the internal control is above C_T 34, this points to a purification problem or a strong positive sample that can inhibit the internal control. In the latter case, the assay is valid. If a water control run is performed, the IC must fall below a C_T of 33.

15 LIMITATIONS OF THE METHOD

The results must always be considered in relation to the clinical symptoms. Therapeutical consequences should be made in consideration of clinical data.

A negative test result does not exclude a *Borrelia burgdorferi* infection.

16 TROUBLESHOOTING

The following troubleshooting guide is included to help you with possible problems that may arise when performing a real time PCR.

No fluorescence signal in the FAM channel of the positive control

The selected channel for analysis does not comply with the protocol

Select the FAM channel for analysis of the *Borrelia*-specific amplification and the VIC®/HEX/JOE/TET channel for the amplification of the control DNA.

Incorrect configuration of the real time PCR

Check your work steps and compare with chapter "Procedure".

The programming of the thermal profile is incorrect

Compare the thermal profile with the protocol (table 5).

Incorrect storage conditions for one or more kit components or kit expired

Check the storage conditions and the date of expiry printed on the kit label. If necessary, use a new kit and make sure kit components are stored as described in chapter "Transport, Storage and Stability".

Weak or no signal of the control DNA and simultaneous absence of a signal in the bacteria-specific FAM channel.

real time PCR conditions do not comply with the protocol

Check the real time PCR conditions (table 5).

real time PCR inhibited

Make sure that you use an appropriate isolation method (see chapter "Sample Preparation") and follow the manufacturer's instructions. Make sure that the ethanol-containing washing buffer of the isolation kit has been completely removed. An additional centrifugation step at high speed is recommended before elution of the DNA.

DNA loss during isolation process

In case the control DNA was added before extraction, the lack of an amplification signal can indicate that the DNA isolation was not successful. Make sure that you use an appropriate isolation method (commercial kits are recommended) and stick to the manufacturer's protocol.

Incorrect storage conditions for one or more components or kit expired

Check the storage conditions and the date of expiry printed on the kit label. If necessary, use a new kit and make sure kit components are stored as described in chapter "Transport, Storage and Stability.

Detection of a fluorescence signal in the FAM channel of the negative control

Contamination during preparation of the PCR

Repeat the real time PCR in replicates. If the result is negative in the repetition, the contamination occured when the samples were pipetted into the optical PCR reaction tubes. Make sure to pipet the positive control last and close the optical PCR reaction tube immediately after adding the sample. If the same result occurs, one or more of the kit components might be contaminated. Make sure that work space and instruments are decontaminated regularly. Use a new kit and repeat the real time PCR.

17 KIT PERFORMANCE

17.1 Diagnostic sensitivity and specificity

During the validation study of the MutaPLEX® Borrelia real time PCR, 48 positive and 120 negative samples were tested. The diagnostic sensitivity was found to be 100 % and the diagnostic specificity 100 %

The positive predictive value was found to be 100 %, the negative predictive value showed to be 100 %.

Table 8: Overview of the amount of samples tested and the resulting positive and negative predictive values

	positive samples	negative samples
MutaPLEX® Borrelia positive	48	0
MutaPLEX® Borrelia negative	0	120
Sensitivity	100 %	
Specificity	100 %	

17.2 Analytical Sensitivity

The limit of detection (LoD) of the MutaPLEX® Borrelia real time PCR TM kit was determined using serial dilutions of *Borrelia burgdorferi* in culture medium in a Stratagene Mx3000 real time PCR instrument. Total nucleic acids were extracted using MutaCLEAN® Universal RNA/DNA (KG1038) according to the manufacturer´s instructions. Each sample was supplemented with 5 µl control DNA prior to extraction. Total nucleic acids were eluted with 50 µl and 4 µl of the eluates were applied to the subsequent real time PCR.

The LoD of the MutaPLEX® Borrelia real time PCR TM kit for *Borrelia burgdorferi sensu lato* is >10 genome copies per reaction each.

The sensitivity of the MutaPLEX® Borrelia real time PCR TM kit was also analysed by testing round robin samples of known status.

All samples of the QCMD *Borrelia* panels were detected correctly. Likewise the samples of the *Borrelia* ring trial (INSTAND e. V.). Results are shown in table 9.

Table 9: Samples tested for the validation of the sensitivity of the MutaPLEX® Borrelia real time PCR TM kit.

Sample	Sample content	Expected Result	MutaPLEX® Borrelia	Sample type
BbDNA14-01	*Borrelia garinii*	positive	positive	core

Sample	Sample content	Expected Result	MutaPLEX® Borrelia	Sample type
BbDNA14-07	*Borrelia garinii*	positive	positive	core
BbDNA14-08	*Borrelia garinii*	positive	positive	educational
BbDNA14-09	*Borrelia burgdorferi s.s.*	positive	positive	core
BbDNA14-03	*Borrelia burgdorferi s.s.*	positive	positive	core
BbDNA14-04	*Borrelia burgdorferi s.s.*	positive	positive	educational
BbDNA14-10	*Borrelia afzelii*	positive	positive	core
BbDNA14-05	*Borrelia afzelii*	positive	positive	core
BbDNA14-02	*Treponema phagedenis*	negative	negative	core
BbDNA14-06	*Borrelia negative*	negative	negative	core
1515351	*Borrelia miyamotoi*	negative	negative	–
1515352	*Borrelia bavariensis*	positive	positive	–
1515353	*Borrelia garinii* Ospa Type 8	positive	positive	–
1515354	*Borrelia kurtenbachii*	positive	positive	–

17.3 Analytical Specificity

The specificity of the MutaPLEX® Borrelia real time PCR was additionally evaluated with different other relevant viruses and bacteria found in clinical samples.

Results: The MutaPLEX® Borrelia real time PCR TM kit showed a positiv result for the sample containing *Borrelia burgdorferi*, whereas samples containing other pathogens were reliably tested negative. The results are shown in table 10.

Table 10: Bacterial and viral pathogens tested for the determination of the analytical specificity of the MutaPLEX® Borrelia real time PCR TM kit.

Strain	Expected result	Result
Enterovirus 68	negative	negative
Coxsackievirus B3	negative	negative
Coxsackievirus A16	negative	negative
Coxsackievirus B5	negative	negative
Influenza virus A A/ Brisbane H1N1 59/2007 E40/08	negative	negative
Influenza virus A Indonesia H5N1 05/2005	negative	negative

Strain	Expected result	Result
Influenza virus A Panama H3N2 2007/99	negative	negative
Influenza virus B B/ Brisbane 60/2008 E09/09	negative	negative
FSME virus	negative	negative
Ehrlichia chaffeensis	negative	negative
Ehrlichia ewingii	negative	negative
Ehrlichia canis	negative	negative
Ehrlichia phagocytophilum	negative	negative
Anaplasma platy	negative	negative
Babesia divergens	negative	negative
Babesia microti	negative	negative
Babesia sp. EU1	negative	negative
Borrelia burgdorferi Strain 4681	positive	positive
Borrelia burgdorferi sensu stricto	positive	positive
Borrelia afzelii	positive	positive
Borrelia garinii	positive	positive
Borrelia spielmanii	positive	positive
Borrelia bavariensis	positive	positive
Borrelia bisettii	positive	positive
Borrelia lustianae	positive	positive
Borrelia valaisiana	positive	positive
Borrelia kurtenbachii	positive	positive
Borrelia japonica	negative	negative
Borrelia miyamotoi	negative	negative
T. phagedenis	negative	negative
Leptospiren	negative	negative

18 ABBREVIATIONS AND SYMBOLS

DNA	Deoxyribonucleid Acid	REF	Catalog number
PCR	Polymerase Chain Reaction	→REF	To be used with
REACTION MIX	Reaction Mix		Contains sufficient for <n> test
CONTROL +	Positive control		Upper limit of temperature
CONTROL –	Negative control		Manufacturer
CONTROL DNA IC	Control DNA		Use by YYYY-MM-DD
LOT	Batch code		Consult instructions for use
CONTENT	Content	IVD	In vitro diagnostic medical device

19 LITERATURE

1. Wilking, H. et al. Antibodies against Borelia burgdorferi sensu lato among adults, Germany 2008 – 2011. CDC Emerging Infectious Diseases 21, 1, 2015.
2. Wilking H, Stark K. Trends in surveillance data of human Lyme borreliosis from six federal states in eastern Germany, 2009–2012. Ticks Tick Borne Dis. 2014; 5:219–24

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